A 1000-L SCALE-UP FERMENTATION OF *ESCHERICHIA COLI* CONTAINING PVSEOP7 FOR PRODUCTION OF ORGANOPHOSPHORUS HYDROLASE

Dennis C. Lukens, Vipin K. Rastogi, Tu-c. Cheng, Joseph J. DeFrank, and James J. Valdes
U.S. Army Edgewood Chemical Biological Center, APG, MD 21010

Michael H. Kim
Geo-Centers, Inc., Gunpowder Branch, P. O. Box 68, APG, MD 21010

ABSTRACT

Four 20-L scale batch fermentations in LB medium were performed to investigate time-dependent yields of organophosphorus hydrolase (OPH) in E. coli XL1 containing pVSEOP7. The specific OPH activity was monitored during each 20-L batch fermentation culture by assaying several times between the IPTG induction point (OD of 0.4 to 0.5 at 600 nm) and the cell harvest point (near-stationary phase of each 20-L batch culture). From the four 20-L batch fermentations, only the 20-L batch cultures with 1 mM of cobalt chloride added 3-hr prior to cell harvest resulted in a drastic increase in the specific OPH activity (369 to 1809 U/mL). A similar strategy of 1 mM cobalt chloride addition was successfully used to obtain a 20.7 mg OPH per liter of culture at the 1000-L scale batch fermentation in LB, as compared to 5 to 10 mg/L in flask cultures. The specific OPH activity was shown to be stable through additional process time associated with downstream processing steps such as cell concentration by continuous disk centrifugation, cell paste preparation by bottle centrifugation, cell disruption by Microfluidizer® homogenization, and lysed cell clarification by 0.22 μm tangential flow microfiltration.

INTRODUCTION

Proliferation of neurotoxic chemical warfare (CW) agents in the hands of extremist terrorist[1,2,3] organizations and rogue nations in recent years has significantly increased the prospect of intentional or accidental exposure of civilian populations and military personnel. The currently fielded universal decontaminant, DS2, is highly corrosive and poses serious environmental concerns. Decontamination based on the use of catalytic enzymes offers obvious advantages, i.e. they are non-toxic, non-corrosive, and environmentally safe. Two CW-degrading enzymes, OPH (organophosphorus hydrolase) from *Pseudomonas diminuta* and *Flavobacterium sp*, and OPAA (organophosphorus acid anhydrolase) from *Altermonas JD6.5* have been cloned and expressed in *Escherichia coli* strains.

OPH is the best-characterized bacterial enzyme capable of degrading a variety of chemical warfare nerve agents (CWA), especially highly toxic V-type, VX and Russian-VX. In addition to V-type, OPH is also capable of detoxifying G-type CW agents, agricultural pesticides, and CW agent surrogates such as diisopropylfluorophosphate (DFP). While the OPH enzyme hydrolyzes paraoxon at a near diffusion-rate

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
19 NOV 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A 1000-L Scale-Up Fermentation Of Escherichia Coli Containing Pvseop7 For Production Of Organophosphorus Hydrolase	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Edgewood Chemical Biological Center, APG, MD 21010**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003. , The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

(k_{cat} > 3,500 s^{-1}), the VX is hydrolyzed at a very low rate (k_{cat} > 0.3 s^{-1}). Development of a broad-spectrum enzyme-based decontaminant for CW agents relies on inclusion of OPH in addition to the other well-characterized microbial enzyme such as OPAA.

Large-scale availability of OPH and other CW agent degrading enzymes, i.e. OPAA[4,5,6], is a pre-requisite to the development of an enzyme-based decontamination system for detoxifying a variety of CW nerve agents and toxic pesticides. With a non-optimized, single-batch incubation of cells in a shaker flask, typical OPH yields range from 5 to 10 mg/L culture when OPH gene is expressed under a high-expression promoter. Here, we report the studies on several 20-L and scaled-up 1000-L fermentation runs performed with recombinant *Escherichia coli* cells harboring pVSEOP7 plasmid expressing OPH.

MATERIAL AND METHODS

1. BACTERIAL STRAIN SOURCE

Plasmid pJK33, containing a near-full length OPH gene from *Flavobacterium* was kindly provided by W. Mulbry, USDA, Beltsville, MD. The full-length OPH gene was re-designed and sub-cloned into pSE420 (Invitrogen) and transformed into XL1 strain of *Escherichia coli*. The recombinant cell line harboring pVSEOP7 was used in the present study.

2. GROWTH MEDIUM AND CONDITIONS

The medium used for all the fermentations was Luria Broth (LB, Gibco BRL). The cultures were shaken at 225 rpm and grown at 30°C.

3. FERMENTATION RUNS

3.1 20-LITER FERMENTATION

Two vials of frozen glycerol stocks (2 ml each) of XL1 (pVSEOP7) were used to inoculate 500 ml of LB medium supplemented with 100 μg/ml ampicillin (Na salt, Fisher Scientific) in a 2-L flask. The 2-L flask was incubated at 30°C and 225 rpm in an incubator shaker (Innova 4300, New Brunswick Scientific). As cell growth reached the OD of 0.4-0.6 absorbance at 600 nm, the 500 ml culture was used to inoculate about 20 L media in the 30-L Micros fermentor (New Brunswick Scientific). The media composition in the Micros fermentor was as follows: 500 g LB, 20 kg deionized water, 20 ml ampicillin Na salt (100 mg/ml, Fisher), and 1 ml Antifoam 204 (Sigma). Sterile antifoam and pre-filtered (0.2 μm, Nalgene) ampicillin solutions were aseptically added after autoclaving at 121°C for 30 min and cooling to the operating temperature of 30°C. The operating conditions for the Micros fermentor were as follows: 30°C, 240 rpm, 20 L/min airflow rate, and 1 psig overhead pressure. The pH was controlled with the addition of 3 M phosphoric acid (Fisher Scientific). When the A_{600} inside the Micros fermentor reached 0.4 to 0.6, the cultures were induced with 20 ml of pre-filtered 1 M isopropyl β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested approximately 15 hrs after the IPTG induction. In one batch 20-L fermentation, 10 ml of pre-filtered 1 M $CoCl_2$ (Sigma) was added 3.5 hrs prior to cell harvesting. For each 20-L fermentation, process temperature, pH, airflow rate, overhead pressure, mixing speed were monitored, logged and controlled by a ML-4100 multi loop microprocessor controller (New Brunswick Scientific) and BioCommand 2.62 (New Brunswick Scientific). DO level was only monitored and logged. At each data point time interval, approximately 1 g of wet cells was collected in a 50-ml centrifuge tube after centrifugation (Beckman J2-21M) at 15000 rpm for 10 minutes and used for cell disruption and crude extract OPH activity/protein analysis.

3.2 1000-LITER FERMENTATION

A loopful frozen stock of pVSEOP7/ XL1 blue was streaked on a LB agar plate supplemented with 100 ug/ml ampicillin and incubated at room temperature over a weekend. A 5 ml LB suspension media in a test tube supplemented with 100 μg/ml ampicillin was inoculated with five colonies (the sizes of colonies were too small) from the LB-Amp plate and incubated at 37°C with shaking at 225 rpm. After 4.5 hours of incubation, a 5-ml aliquot of culture was used to inoculate 250 ml LB containing 100 μg/ml ampicillin at 30°C and 225 rpm for an overnight growth. The 250 ml overnight culture was subsequently used to inoculate the 20-L seed fermentor. The same LB medium and operating conditions as the previous Micros fermentor runs were used in the 20-L seed fermentor except no pH control. When the A_{600} of the seed fermentor (~21 L) reached 0.85, the culture was aseptically transferred to the IF 1500 fermentor (New Brunswick Scientific) through a sterile transfer hose. The composition of the IF 1500 fermentor was as follows: 25.0 kg LB, 1000 kg DI water, 1000 ml ampicillin Na salt (100 μg/ml), and 50 ml Antifoam 204. Pre-filtered (0.2 μm) ampicillin and antifoam solutions were aseptically added after autoclaving at 121°C for 60 minutes and cooling to the operating temperature of 30°C. The operating conditions for the IF 1500 fermentor were as follows: 30°C, 110 rpm, 1000 L/min airflow rate, and 1 psig overhead pressure. The pH was controlled at 7.3 with the addition of 3 M phosphoric acid. When the A_{600} inside of the IF 1500 fermentor reached 0.39, the culture was induced with 1000 ml of pre-filtered (0.2 μm) 1 M IPTG. Approximately 11 hours after the IPTG induction, 1000 ml of pre-filtered (0.2 μm) 1 M $CoCl_2$ was added to the IF 1500 fermentor. Three additional hours were allowed for cobalt incorporation into expressed OPH prior to setting the IF 1500 fermentor to maximum cooling for cell harvesting. Process temperature, pH, airflow rate, overhead pressure, and mixing speed were monitored, logged and controlled by a ML-4100 multi loop microprocessor controller and BioCommand 2.62. DO level was only monitored and logged.

4. CELL HARVEST

The cells were initially harvested with BTPX Disk Centrifuge (Alfa Laval) into a cell slurry and further centrifuged into cell paste by Beckman J2-21M. The wet cells collected are represented as g wet cells/L culture. The cell paste was stored at –80°C.

5. CELL DISRUPTION AND LYSED CELL CLARIFICATION

The frozen cell paste was thawed overnight at 4°C and resuspended in BTP (bis-tris propane) buffer (10 mM BTP, 0.1 mM $CoCl_2$, pH 7.4) in a 1 kg: 4 L ratio by a high speed blender at ~4°C. The resuspended cell solution was further filtered in-line through a 150-mesh screen followed by cell breaking in Microfluidizer® (Microfluidics). The feed to the Microfluidizer® was maintained at 4°C and the lysed cells were collected in an iced container. The average break-pressure was about 16000 psig. The cell debris was removed through a 0.22 μm Pellicon 2 tangential flow filter unit (Millipore) with a total surface area of 5 ft^2.

6. OPH ENZYME ACTIVITY ASSAY

The OPH assays were performed in 50 mM CHES buffer (pH 9) at room temperature using paraoxon (Chem-Serve) as substrate at a concentration of 0.6 mM. The hydrolysis rates were calculated using ε^{400}=13,500 M^{-1} cm^{-1}. A 1-ml assay solution contained 10 μl diluted enzyme preparation, 10 μl 60mM paraoxon (in methanol) and CHES buffer. Substrate spontaneous hydrolysis rates were also measured and subtracted from the observed enzymatic rates. OPH specific activity was defined as μmoles (U) substrate hydrolyzed/min/mg protein.

RESULTS AND DISCUSSION

1. FERMENTATION RUNS

1.1 20-LITER SEED PREPARATION

Growth characteristics of four shake-flask cultures of recombinant *E. coli* XL1 Blue (pVSEOP7) in LB are shown in Figure 1. Slight growth lags were evident in the runs #1 and #2, and longer culture times were required for the runs #3 and #4 to reach the A_{600} values of 0.4 to 0.6. These different growth results could be attributed to two separate sets of the 2-ml frozen stock vials used for the seed preparation. Nevertheless, the average specific growth rates (μ) ranged between 0.55 and 0.62 hr^{-1} during the exponential phases, indicating consistent characteristics of all four seed cultures.

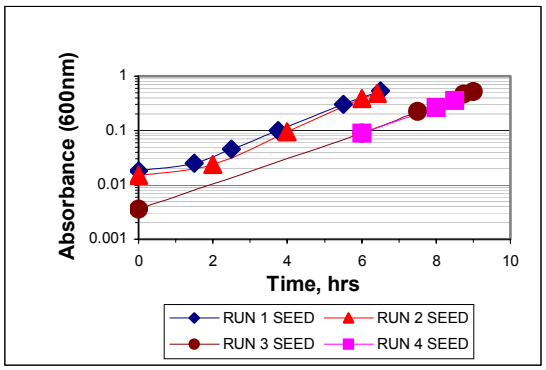

Figure 1. Characteristics of four 500-ml seed cultures.

1.2 20-LITER FERMENTATIONS

Growth characteristics of *E. coli* XL1 (pVSEOP7) in the 20-L fermentor were reproducible from one batch run to another. All the 20-L batch cultures grew exponentially soon after inoculation, with growth rates ranging from 0.53 to 0.58 hr^{-1}, and the final A_{600} values at stationary phases were approximately 4.3 for the four batch 20-L fermentation runs. The DO value in the media was consistent from batch run-to-batch run. Initially, the DO gradually decreased to 70% around the culture times of 11 to 12 hours, coinciding with a DO demand associated with exponential culture growth.

As the cultures approached the stationary phase, the DO increased to about 95%, and the DO availability never became a limiting factor during the batch cultures of XL1 Blue in LB medium. The residual DO level of 95% could be due to some residual endogenous activities. The pH control at 7.0 to 7.2 was used in all the 20-L batch fermentations except the first run where no pH control was used. Without pH control, the pH of the culture continuously increased and reached 8.3 overnight in the first 20-L batch fermentation.

The expression level of OPH was monitored by assaying OPH activities in each 20-L batch fermentation run toward the end of batch culture period (Figure 2). Only one 20-L fermentation run (#4) resulted in enhanced OPH yield (369 to 1809 U/ml crude cell extract) when Co^{++} was added to the culture 3 hours prior to cell harvesting (Figure 2).

The other three 20-L fermentation runs without Co^{++} added resulted in the same low-level yield. This result was in contrast to the IPTG induction of a similar culture grown in the succinate media, where a high OPH yield was obtained (data not shown).

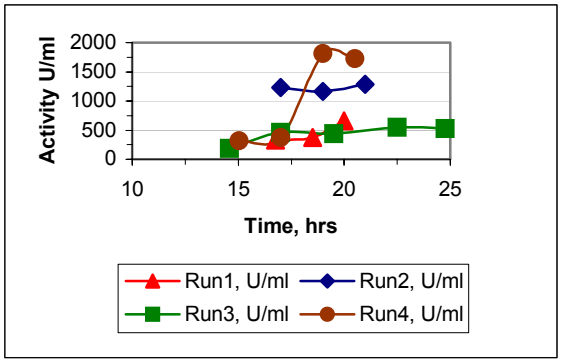

Figure 2. Time-dependent OPH expression level in 20-L fermentation runs.

Nevertheless, based on the 20-L LB fermentation results, it was concluded that a three-hour cobalt incubation prior to cell harvesting is critical for an optimum OPH production.

1.3 1000-LITER FERMENTATION

After the seed culture in the Micros fermentor reached A_{600} of 0.85, it was used for the inoculation of the1000-L fermentor. As expected, *E. coli* XL1 (pVSEOP7) exhibited an exponential mode of growth with a slight lag time immediately after the inoculation into the 1000-L fermentor (Figure 3). Initially, the maximum growth rate was 0.54 hr^{-1} when the cultures were induced with 1 mM IPTG at A_{600} of 0.4. Thereafter, the growth rate continually decreased until the cultures reached the stationary phase.

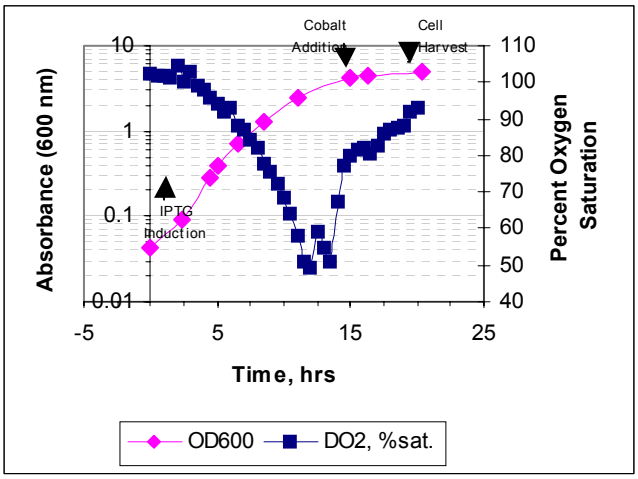

Figure 3. Growth and dissolved oxygen responses in the 1000-L fermentation run.

The timing of a minimum DO in the batch fermentation (50% at 12 hours) and in 20-L runs (70% after 11 to 12 hours) was almost identical to the 1000-L fermentation. The lower DO value (50%) at the 1000-L scale indicated a less efficient mode of oxygen transfer capacity than those of the 20-L scale fermentation runs. To improve oxygen uptake, the mixing speed of 110 rpm could be increased to 150 rpm, but this was not critical since the DO never became a limiting factor in the 1000-L scale fermentation run.

Three hours prior to cell harvesting, 1 mM $CoCl_2$ was added to the final concentration of 1 mM. As previously shown in 20-L fermentation studies, the OPH yield in the 1000-L fermentator increased drastically (from 238 to 2012 U/ml in crude cell lysate; Figure 4). Six hours after cobalt was added, the

OPH yield decreased to 1726 U/ml. The wet cell weight also decreased from 8.0 g/L to 7.82 g/L, indicating the cultures might have entered a late log phase of growth.

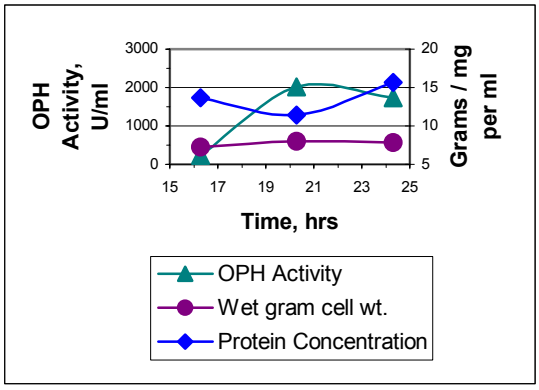

Figure 4. Yield of OPH in the 1000-L fermentation run.

2. CELL HARVESTING

Immediately after setting the IF 1500 fermentor in a maximum cooling mode for cell harvesting, 1000 ml of culture was collected for a wet cell weight estimation (approximate 9 wet g/L). A total of 12.4 L cell slurry, pooled from the disk centrifuge, was collected and was further centrifuged at 14000 rpm for 15 minutes in 240 ml bottles. A total of 5.97 kg wet cell paste was obtained and packed in polypropylene containers for storage at –80^{o}C.

3. CELL DISRUPTION AND LYSED CELL CLARIFICATION

The frozen cell paste (5.97 kg) was thawed overnight at 4^{o}C and resuspended in 24 L BTP buffer (10 mM BTP, 0.1 mM $CoCl_2$, pH 7.4) by a high-speed blender while maintaining the suspension on ice. The resuspended cell solution was further filtered in-line through a 150-mesh screen prior to using the Microfluidizer®. The average break pressure was set around 16000 psig. The feed to the Microfluidizer® was maintained below 4^{o}C, and the lysed cells were collected in an iced container. The lysed cell suspension was filtered through 0.22 μm Pellicon 2 tangential flow filter unit with a total surface area of 5 ft^2. A total of 25.2 L of clear lysate was collected with OPH activity of 15794 U/ml and protein concentration of 20.7 mg/ml. The calculated specific activity of 763 U/mg protein was much higher than previously assayed value of 174 U/mg protein of 20-L run. Three possible factors could contribute to the higher OPH yield: 1) increased cobalt concentration from 0.5 mM final concentration to 1 mM final cobalt concentration, 2) additional time allowed in downstream processing steps for the cobalt incorporation and 3) more effective cell disruption by the Microfluidizer® than the mini French press treatment.

CONCLUSIONS

A 1000-L scale-up fermentation with *E. coli* XL1 blue containing pVSEOP7 in LB was successfully achieved to produce a higher yield of active OPH enzyme compared to that of batch flasks. Approximately, 26.5 mg OPH per liter of culture volume was obtained. The early IPTG induction at A_{600} value around 0.4 and three-hours cobalt incubation prior to cell harvesting at an early stationary phase

were critical for the higher OPH production. Additional downstream process times associated with the 1000-L scale fermentation might have also resulted in the higher OPH recovery. Further studies are recommended to identify the effect of cobalt during cell growth, the refinement of downstream processing steps, and the optimization of OPH expression during cell growth.

REFERENCES

1. Lai, K., Stolowich, N.J. and Wild, J.R. 1995. Characterization of P-S bond hydrolysis in organophosphorothioate pesticides by organophosphorus hydrolase. Archives of Biochemistry & Biophysics 318: 59-64.
2. Kolakowski, J.E., J.J. DeFrank, S.P. Harvey, Szafraniec, L.L., Beaudry, W.T., Lai, K. and Wild, J.R. 1997. Enzymatic hydrolysis of chemical warfare agent, VX and its neurotoxic analogues by organophosphorus hydrolase. Biocatalysis and Biotransformation 15: 297-312.
3. Rastogi, V.K., DeFrank, J.J., Cheng, T.C., and Wild, J.R. 1997. Enzymatic hydrolysis of Russian-VX by organophosphorus hydrolase. Biochem. Biophys. Res. Commun. 241: 294-296.
4. Cheng, T-C., Harvey, S.P., and Chen G.L. 1996. Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. Appl. Environ. Microbiol. 62: 1636-1641.
5. Cheng, T-C., Liu, L., Wang, B., Wu, J. DeFrank, J.J., Anderson, D.M., Rastogi, V.K. and Hamilton, A.B. 1997. Nucleotide sequence of a gene encoding an organophosphorus nerve agent degrading enzyme from Alteromonas haloplanktis. J. Ind. Microbiol. & Biotech. 18: 49-55.
6. Cheng, T-C., Rastogi, V.K., DeFrank, J.J., and Sawiris, G.P. 1998. G-type nerve agent decontamination by Alteromonas prolidase. Annals NY Acad. Sci. 864:253-258.